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RESEARCH ARTICLE

CORRELATION BETWEEN KOILOCYTES AND HUMAN PAPILLOMA VIRUS IN NASOPHARYNGEAL CARCINOMAS

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is classified as a malignant neoplasm and is endemic to the Far East Asia. Oncogenic viruses such as Epstein Barr virus (EBV) and Human papillomavirus (HPV) are known to contribute to the pathogenesis of NPC tumours, with the former being well establish to have a strong association with the cancer. Little is known about the association of HPV, as well as the correlation of cytopathic effect induced by this virus and its presence in NPC tissue samples. The aim of this study was to compare the histopathological features (Koilocytosis) with PCR method to predict the presence of HPV infection in formalin-fixed-paraffin-embedded tissue (FFPET) samples of NPCs. Seventy-two archival (72) NPC tissues samples from the year 2006 to 2012 were retrieved from Pathology Department of University of Ghana School of Biomedical and Allied Health sciences. Sections were taken for histopathological analysis and DNA extraction. Detection of koilocytes was done using light microscopy where tissue samples were regrouped into WHO type I, II and III respectively. Consensus HPV forward primer (GP-E6-3F) and two consensus reverse primers (GP-E7-5B and GP-E7-6B) were used in polymerase chain reaction (PCR) analysis to determine the presence of HPV DNAs in the study sample. Out of the 72 archival NPC biopsies analysed, koilocytes were detected in 22 (30.6%) during microscopy and HPV DNA was detected in 14 (19.4%) during PCR analysis. Ten (10) samples out of the 22 with koilocytes were found to be HPV positive. There was a weak correlation (C= 0.436), though data analysis using Fisher's exact probability test was statistically significant ($p<0.05$). There was a significant level of association between koilocytes and HPV, and thus, the presence of koilocytes in NPC tissues samples can serve as an indicator for the possible presence of HPV in NPC tissue samples.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface and line the nasopharynx, around the ostium of the Eustachian tube within the lateral nasopharyngeal recess or fossa of Rosen Müller (Sham *et al*., 1990; Brennan, 2006; Zeng and Zeng, 2010).

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It differs significantly from other cancers of the head and neck in its occurrence, causes, clinical behavior, and treatment.It is vastly more common in individuals in the Southern region of China and South-East Asia (Chen *et al*., 1990). The highest incidence of NPC are found among the southern Chinese population and in isolated northern populations such as Eskimos and Greenlanders (Parkin, 1997), whiles intermediate incidence is reported in the Mediterranean basin, especially among the Arabic populations of North Africa (Chaouki and el Gueddari, 1991; Benider *et al*., 1995). The etiology of NPC seems to be multifactorial with evidence that genetic, viral (Epstein Bar Virus-EBV and human papillomavirus- HPV),

and other environmental factors are involved (Hildesheim and Levine, 1993). HPV involvement in NPC has been suggested due to the detection of HPV DNA in between 9% and 51% of NPC tissues (Tung *et al*., 1999; Laantri *et al*., 2011; Singhi *et al*., 2012). This is also supported by the fact that HPV shows epitheliotropism and has the ability transform normal human keratinocytes to immortalize forms in vitro (Hwang *et al*., 1993; Wu *et al*., 2000; Francis *et al*., 2000; Goodwin and DiMaio, 2000). The HPV family comprises more than 100 genotypes, classified in accordance with the type of epithelial cells they infect and the ability to affect cellular transformation. Certain types of HPV infect cutaneous epithelial cells, whereas others infect mucosal epithelial cells of the oral cavity, oropharynx, anogenital tract and the uterine cervix (Ragin *et al*., 2007). The genomic HPV DNA has nine open reading frame sequences present on a single strand of DNA and is divided into seven early (E1–E7) and two late phase genes (L1–L2). The expression of oncoproteins E6 and E7 interferes with crucial cellular mechanisms such as cell cycle, regulation and apoptosis (Hummel *et al*., 1992; Kumar *et al*., 2007).

HPV-positive NPC are clinically and molecularly distinct from HPV-negative NPC and has been shown to respond differently to treatment and also have varied prognostic outcomes (Umudum *et al.*, 2005; Atighechi *et al.,* 2014). HPV can be detected by many techniques, including polymerase chain reaction (PCR), southern blot, dot blotting, and *in situ* hybridization (ISH) (Abreu *et al*., 2012). These methods are adequate and reliable for detection of HPV, but are neither easily available universally, nor can be afforded by patients especially in a developing country such as Ghana. While histological examination of haematoxylin and eosin (H&E) stained tissues by light microscopy is the most commonly used method for confirming a NPC diagnosis, and it is also a useful method for the detection of HPV virus (due to cytopathic changes) when molecular biology methods are not available. Based on light microscopy examination, HPV alterations of squamous cells (koilocytes) have been demonstrated in both tissues and cytological smears (Haberland-Carrodeguas *et al.*, 2003; Alves de Sousa *et al*., 2012; Rachmadi *et al*., 2012). Koilocytes are epithelial cells that contain an acentric, hyperchromatic, moderately enlarged nucleus that is displaced by a large perinuclear vacuole (Krawczyk *et al.*, 2008). They represent a cytopathic effect (CPE) of HPV, and is considered to give a clue to the diagnosis of HPV infection. The present study aims to compare the histopathological features (Koilocytosis) with PCR method to predict the presence of HPV infection in NPC biopsies as the detection of HPV in patients with NPC may be of enormous help in planning therapy and also determine prognosis.

MATERIALS AND METHODS

Study Site

This research was a retrospective study to investigate the presence of the DNA of HPV and its association with koilocytes from primary NPC tissue samples. A total of 72 FFPET samples from patients with histopathologically confirmed NPCs from the Pathology Department were used. The tissue samples were archival specimens obtained from the Department of Pathology, University of Ghana School of Biomedical and Allied Health Sciences (SBAHS) Korle-Bu Teaching Hospital, from January 2006 to December 2012. The clinical data on age and diagnosis of the NPC were recorded on each study case. The study was approved by the research and ethical review committee of SBAHS. Permission was also obtained from the Department of Pathology, SBAHS for the use of the archived NPC tissue samples.

Histological Analysis

A 5µm section of the processed paraffin embedded NPC tissues of each specimen was taken using a microtome. The sections were then stained with H & E staining technique as described elsewhere (Bancroft and Gamble, 2008), using an automated staining system (Leica Auto Stainer XL). The H & E stained slides were viewed under light microscope to determine the presence of koilocytes and to confirm the previous diagnosis of the NPC. The histopathological subtypes of NPCs were then determined according to the WHO 1991 classification. It consisted of type I (Keratinizing squamous cell carcinoma), type II (Differentiated non-keratinizing carcinoma) and type III (Undifferentiated non-keratinizing carcinoma).

Molecular Analysis

Genomic DNA Extraction

Ten micrometre (10µm) sections of the tissue were taken using a microtome. To avoid carry-over of the samples and contamination, the microtome blade was changed after each section and all surfaces were also cleaned with xylene after each tissue section. The 10µm sections were placed into a sterile 2.0ml micro-centrifuge tube. The tissues were then digested in 250µl of digestion buffer with pH 8.5 and incubated for 16 hrs at 55^0C, with subsequent proteinase K inactivation at 100˚C for 5 min on a heat-block and allowed to cool to room temperature. The samples were then centrifuged at 13000 rpm for 5 min in a microcentrifuge. The residues containing DNA lysate were pipetted out and the supernatant containing the paraffin discarded. The residue realized (DNA lysate) after the processes contained the DNA extract that was used as a source of DNA template for the PCR analysis as described previously (Dabić *et al*., 2004).

Human β globulin PCR

The DNA extracts were analyzed for the presence of human genomic DNA by human β-globin DNA PCR using the primers; PCO3+ and PCO4+. This PCR analysis served as quality control to ascertain the presence of human genomic DNA in the extract and also the wholesomeness of the DNA in the DNA tissue extract for PCR. The PCR condition was as described by de Roda Husman *et al* (1995) with few modifications using Techne PCR machine (Techne Incorporated, USA). For a total volume of PCR reacting mixture of 25µl, the reaction conditions were as follows; 3µl of 10X PCR buffer (Biopioneer, Co, USA), 1.5mM $MgCl_2$ (Biopioneer Co, USA), 200µM of each of the four oligonucleotide triphosphates (dNTPs) (Sigma, Co, USA), 25 pmol of each consensus primers PCO3 and PCO4 and

0.625units of Taq Polymerase enzyme (Biopioneer Co, USA) and 1µl or 4µl of the DNA lysate; the tissue digested product (source of HPV DNA template). Nuclease free water (Promega Co, USA) was used to make up the volume to 25µl. A master mix was made without the DNA template, vortex to mix and then pulse centrifuged for 30 seconds before aliquoting out into the samples in the PCR tubes. The negative control (25µl of the master mix) and positive control were also taken through the same PCR reaction conditions as per samples. The PCR cycling conditions were as follows; 95ºC for 4min, followed by 40 cycles of 95ºC for 1min, 55ºC for 2min, 72ºC for 1.5 min and final elongation step of 72ºC for 4 min (Sotlar *et al*., 2004)**.** After the reaction, 10µl of the PCR product was then separated on 2% agarose gel (Biopioneer Co, USA) in 1X TAE buffer (Biopioneer Co, USA) through gel electrophoresis at 80 volt (Labnet International, Power station 300) using 2µl of blue/orange DNA loading dye (6X) (Promega Co, USA) and stained with 0.5µg/ml ethidium bromide (Life Technologies Co, USA). Hundred base pair nucleotide sequence molecular size marker (Sigma Mo, USA) was run alongside the PCR products on the gel. The gel was photographed using UV-illumination (UVI save gel documentation system, model GAS9200/1/2/3, Version 12) and the picture was then analyzed after the end of electrophoresis.

PCR Analysis

The HPV in the NPC tissue biopsies were obtained by using conventional PCR as previously describe (Sotlar *et al*., 2004), with few modifications. In the PCR, 4µl of DNA lysate obtained after the tissue digestion were used as a source of HPV DNA template, to amplify a 630bp region in the E6/E7 region of the HPV genome using HPV general primers. In the PCR amplification reaction, a single consensus forward primer (GP-E6-3F) and two consensus reverse primers (GP-E7-5B and GP-E7-6B) were used, using Techgene PCR machine. For a total volume of PCR reaction mix of 25µl the reaction conditions was as follows; 3µl of 10X PCR buffer (Biopioneer, Co, USA), 1.5mM MgCl2 (Biopioneer Co, USA), 200µM of each of the four oligonucleotide triphospates (dNTPs) (Sigma, Co, USA), 3.75 pmol of each consensus primers and 0.625units of Taq Polymerase enzyme (Biopioneer Co, USA) and 1µl or 4µl of the DNA lysate; the tissue digested product (source of HPV DNA template). Nuclease free water (Promega Co, USA) was used to make up the volume to 25µl . A master mix was made without the DNA template, vortex to mix and then pulse centrifuged for 30 seconds before aliquoting out into the samples in the PCR tubes. The positive control was HPV18 DNA-containing plasmid and negative control (25µl of primer mix). The controls were also taken through the same PCR reaction conditions as per samples. The PCRs were performed by the following cycling conditions; an initial denaturation at 94ºC for 4 min, followed by 40 PCR cycles consisting of 1min denaturation step at 94ºC, annealing step at 40ºC for 2min and elongation step at 72ºC for 2min. Then, a single final elongation step at 72ºC for 10min. As an internal control for the PCR work, the positive and the negative control PCR products were analyzed on a 2% agarose gel for every batch of the PCR run.

Analysis of PCR Amplifications

The amplification products were analysed by gel electrophoresis on 2% agarose gel and stained with 0.5µg/ml ethidium bromide. Ten microlitres of each sample was added to 2µl of orange G (5X) gel loading dye for the electrophoresis. Hundred base pair DNA molecular weight marker (Sigma, MO, USA) was run alongside the PCR products. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system at 120 volts for 40 min and was optically visualized and photographed over a UV trans-illuminator (UVIsave gel documentation system, model GAS9200/1/2/3, Version 12) and the pictures were then analyzed.

Microscopic Analysis for Koilocytosis

Microscopic analysis of NPC tissue samples for the presence of koilocytes was done using the binocular light microscope, OLYMPUS 500 microscope (Version E_LCmiro_09Okt, 2009), at the Department of Biomedical and Forensic Sciences, University of cape Coast. Each tissue sample was observed under the microscope for the presence of koilocytes. To confirm a tissue positive for koilocyte under the microscope, the following tissue characteristics were looked out for; nuclear enlargement, irregularity of the nuclear membrane contour, highly basophilic nucleus and a perinuclear halo found specifically within squamous cells in the tissue section. Tissues positive for koilocytes were further analysed employing the digital camera of the OLYMPUS 500 microscope, which was fitted on the microscope and an LCmicro imaging software on the computer.

Statistical Analysis

The data obtained were analyzed using Statistical Package for Social Sciences (SPSS) version 16 (Chicago, IL). Association between the presence of koilocytes and HPV infection were determined by Fishers' exact test with corresponding 95% confident interval (CI) and Fisher' exact test's probability value (p value) was used to measure the statistical significance of the calculated odds ratio (OR). The P value obtained as is deemed statistically significant when value of the corresponding association is less than or equal to 0.05. Ethical approval for the study was granted according to the ethics and guidelines established by the College of Health Sciences, Accra, Ghana, with ethical clearance protocol identification number: MS-Et/M.7-P 4.6/2012-13.

RESULTS

The demographic characteristics of the NPC cases (72) showed the mean age of patients to be 36.33 years ±SD= 20.82, with age ranging from 10-79 years. Morphological criteria suggestive of HPV infection was confirmed by microscopy by the detection of koilocytes (Figure 1) in the squamous cells and molecular by PCR analysis (Figure 2) of the NPCs examined in this study. The distribution of koilocytes in the NPC histopathological subtypes showed that out of the total 22(30.6%) koilocytes observed, 2 (2.8%) were positive for type I, 4 (5.6%) were of the differentiated non-keratinized (type II), whiles 16(22.2%) were of the type III, of the total 72 NPC cases (Table 1).

In table 2, the overall prevalence of HPV in the studied NPC tissue samples was 19.44% (14 out of 72). Out of the 14 HPV DNA positive cases detected, 10 were of the undifferentiated (type III); 3 were of the differentiated non-keratinized (type II) and only 1 was of the keratininized type (type I). Considering the 58 HPV negative NPC cases identified, 46 were type III, 10 were type II, and 2 were of the type I.

Table 1. Koilocytes Distribution in the NPC Subtypes

(*n*=72)

	NPC WHO Subtypes			
Count Koilocytes	Type 1	Type 2	Type 3	Total
Present	2 (2.8%)	4(5.6%)	16(22.2%)	22(30.6%)
Absent	1 (1.4%)	9(12.5%)	40(55.6%)	50(69.4%)
Total	3(4.2%)	13(18.1%)	56(77.8%)	72(100%)

KEY: n=Number of cases; Values are expressed as n (%);

Table 2. Distribution of HPV in NPC histological subtypes

(*n*=72)

NPC type	HPV+ (%)	HPV- (%)	Total
WHO type 1	1 (1.39)	2 (2.78)	3 (4.17)
WHO type 2	3 (4.16)	10 (13.89)	13 (18.06)
WHO type 3	10 (13.89)	46 (63.89)	56 (77.78)
Total	14 (19.44)	58(80.56)	72 (100)

KEY: n=Number of cases; Values are expressed as n (%);
HPV-: HPV negative NPC cases; HPV+: HPV positive NPC cases

Table 3, shows the odds ratio (OR) for koilocytic NPC positive HPV and koilocytic NPC negative HPV cases, with corresponding 95% confidence interval among the 72 NPC tissue samples under study.

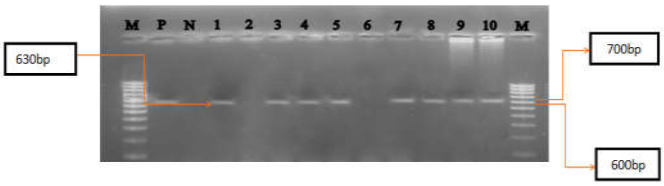

Figure 2. An electrophoregram of PCR products of human papillomavirus. The 630bp fragment corresponds to the amplified HPV DNA. Lane M: 100bp molecular size marker; P: positive control; N:negative control (no DNA);1,3,4,5,7,8,9,10: HPV DNA positive samples; 2 and 6: HPV DNA negative samples.

HPV positive NPCs who accounted for 10 (45.5%) of the koilocytic NPC tumor cases, were more likely to have koilocytes in the NPC tissue sections (OR=9.5; 95% CI=2.55-35.95) as compared to the HPV negative NPC cases, eventhough the later had higher proportion [12 (54.5%) out of 22] of the koilocytes in the tumor cases.

Table 3. Correlation between Koilocyte and HPV (*n*=72)

Parameters	Absent (%)	Present (%)	OR (95% CI)	*p-value*	C
Koilocytosis/ +HPV	4 (8 %)	10 (45.5 %)	9.5 (2.55-35.95)	0.001	0.436
Koilocytosis/ –HPV	46(92 %)	12 (54.5%)			
Total	50(100%)	22(100%)			

Fisher's Exact Probability Test, p<0.05, was considered statistically significant; C: Correlation; OR: Odds ratio

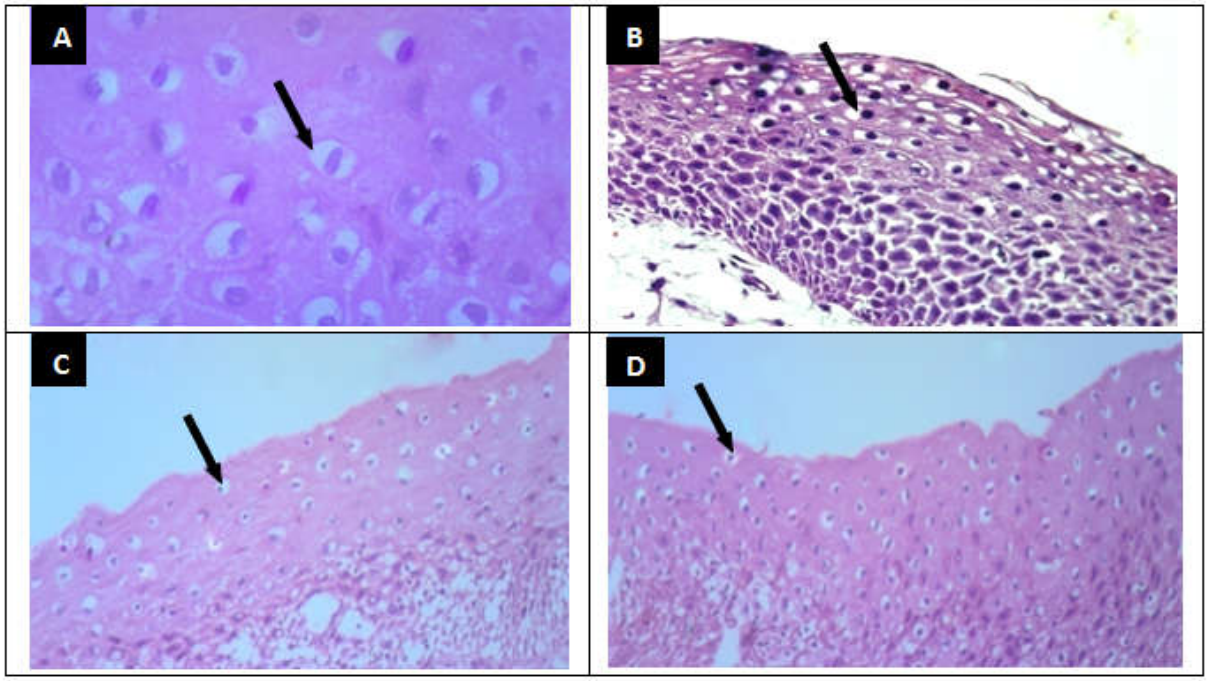

Figure 1. Photomicrographs of HPV positive NPC tissue sections, showing koilocytic features (halos appearing around the nucleus) suggestive of HPV infection (Black arrows) (A) High power magnification (H&E ×400) of malignant squamous cells of NPC (B) High power magnification (H&E ×400) of malignant squamous cells of NPC (C) Lower power magnification (H&E ×100) of malignant squamous cells of NPC (D) Lower power magnification (H&E ×100) of malignant squamous cells of NPC

Thus, there is a 9.5 high chance of finding koilocytes in squamous cells of HPV positive NPC tissue sections histologically, than in the HPV negative NPCs in our research work. The difference between the two parameters were statistically significant (p=0.001), with a corresponding weak correlation (C) of 0.44.

DISCUSSION

All the 72 archival tumour samples were microscopically confirmed by the H and E staining processes to contain malignant cells and were successfully regrouped under the WHO type I, II and III of the NPC classification. From the human β-globulin PCR analysis, all the 72 NPC samples were positive for human β-globulin DNA. Hence all the DNA extracts from the FFPET archival NPC samples used in this study were suitable for PCR analysis (de RodaHusman *et al*., 1995). The histological diagnosis of HPV presence in a given tissue sample, is based on HPV related histopathological observations, such as koilocytes, dyskeratosis, papillomatosis, hyperkeratosis, acanthosis, and parakeratosis. Koilocytes consisting of the presence of abnormal cells that are vacuolated, with nucleus showing pyknosis and large clear perinuclear halos that usually occupies a greater volume than that of the cytoplasm, is the most common HPV related CPE and is considered by pathologists to be the major histopathological feature for determination of HPV infection in histological sections (Fornatora *et al*., 1996). It is considered as a pathognomonic sign of HPV associated lesion. Therefore in this study koilocytosis was used as the morphological criteria for HPV detection.

In endemic areas, the majority of the NPCs are classified as non-keratinizing or undifferentiated type III tumours (DeMay, 2007). In contrast, in non-endemic countries, up to 50% of tumors are of the well differentiated squamous cell variant (Peters *et al*., 1988). Out of the total 3(4.2%) type I cases, 2 (2.8%) were koilocyte positive, which is partly in agreement with research work done in non-endemic areas, where HPV detection in NPC type I is highest (50%) compared to the other subtypes (Punwaney *et al*., 1999), though by proportion, HPV (10 out of 14 HPV positive cases) and koilocyte (16 out of 22 positive koilocytes) were highest in the type III of our research. Out of the 72 archival NPC biopsies screened, HPV DNA was detected in 14 (19.44%) after the PCR analysis and correlation between the morphological criteria suggestive of HPV (koilocytosis) and the presence of HPV confirmed by PCR in the squamous cell carcinoma examined in this study showed a positive correlation of 0.44 which was significant ($p<0.05$), with an odds ratio of 9.5, thus, high (9.5 times) chance of finding koilocytes in squamous cells of HPV positive NPC tissue sections, than in the HPV negative NPCs. This finding is comparable to other research work that correlated PCR-based HPV detection and histopathological findings in cervical cancer biopsies (88.89%) (Salvia *et al*., 2004). In contrast to our work, other research work showed no statistical significance between HPV and koilocytes in oral and oropharyngeal squamous cell carcinomas biopsies (Miyahara *et al*., 2011). Similarly, work done by Löning *et al*., in 1987, who observed koilocytosis in 10 out of 22 squamous cell carcinomas (SCCs) and corresponding 8 HPV positive PCR in the 22 SCCs comprising of carcinomas of the oral cavity, pharynx and the larynx (Löning *et al*., 1987). Other researchers elsewhere have reported evidences of koilocytosis in cytological smears employing light microscopy (Alves de Sousa *et al*., 2012; Rachmadi *et al*., 2012; Gafar *et al*., 2013). Thus, the Fisher's exact value obtained in our work, showed a significance degree of association between koilocytes and the HPV infections and as such, seems to be very important cytopathic sign of HPV presence, with previous reports (Salvia *et al*., 2004; Krawczyk *et al*., 2008; Aggarwal *et al*., 2009) giving credence to this finding.

Conclusion

Results obtained indicates that the presence of koilocytes found in the NPC tissue samples may serve as a possible indicator for the presence of HPV in the tumor. Therefore, the results obtained suggest that in HPV infections, the presence of koilocytes in SCC of NPCs, does to a larger extent, indicate viral infection. A definitive conclusion cannot be pinched from the results obtained and thus warrants further research to be carried out to confirm it.

Conflict of Interest

None declared.

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